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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Vascular endothelial cell growth inhibitor (VEGI), a member of the TNF family, is an endothelial cell-specific inhibitor of angiogenesis. Overexpression by cancer cells of a secretable VEGI fusion protein resulted in abrogation of xenograft tumor progression, but overexpression of full-length VEGI was completely without effect. This finding indicates that secretion is essential for VEGI action. Here we report the identification of two new VEGI isoforms consisting of 251 and 192 amino acid residues. Both isoforms show endothelial cell-specific expression and share a C-terminal 151-residues segment with the previously described VEGI, which comprises 174 residues. The isoforms are generated from a 17 kb human gene by alternative splicing. Their expression is regulated by TNF-α and interferon-γ. VEGI-251, the most abundant isoform, contains a putative secretion signal. VEGI protein is detected in conditioned media of endothelial cells and VEGI-251-transfected mammalian cells. Overexpression of VEGI-251 in endothelial cells causes dose-dependent cell death. VEGI-251-transfected cancer cells form xenograft tumors of reduced growth rate and microvessel density compared with tumors of empty vector transfectants. These findings support the view that VEGI may function as an autocrine inhibitor of angiogenesis and a naturally existing modulator of vascular homeostasis.

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INTRODUCTION

VEGI has also been shown to induce apoptosis in endothelial cells (5), which suggests a rather complex role in vascular homeostasis. In a mature vasculature, the processes of repair during wound healing and the female reproductive cycle will invoke physiologic neovascularization (6, 7). In addition, angiogenesis accompanies and supports the exacerbation of a variety of disorders, including psoriasis, rheumatoid arthritis, and tumor growth (7). Angiogenesis itself requires a number of sequential steps: destabilization of an existing vasculature, endothelial cell proliferation, endothelial cell migration, and structural reorganization of the new vasculature (6, 8). This vascular destabilization, which involves cellular apoptosis, is important for vascular remodeling (9–11), and has been demonstrated to precede tumor neovascularization (12). Although it is becoming clearer that angiogenesis is regulated by both positive and negative regulatory factors (7, 8), the precise mechanism underlying angiogenic control is still presently poorly understood. Studies of an endothelial-specific cytokine, such as VEGI, may help elucidate the role of antiproliferative factors in physiological and pathological angiogenesis.

The previously described VEGI gene product is a protein of 174 amino acids (1). Similar to most TNF family members, hydrophobicity analysis predicts VEGI-174 to be a type II membrane-bound protein with residues 29–174 comprising the extracellular domain. Because TNF family members, such as TNF-α (13) and Fas ligand (14), are cleaved from the membrane to function as soluble proteins, a similar mechanism was assumed for VEGI. Overexpression of full-length VEGI-174 in cancer cells was ineffective on tumor growth (1), which suggests that VEGI-174 is retained by the cancer cells. However, a fusion protein comprising the 28-amino acid secretory signal of interleukin-6 and the putative extracellular domain of VEGI (sVEGI) was found to be able to inhibit tumor growth when overexpressed in cancer cells (1). Recombinant VEGI comprising only the putative extracellular domain was shown to be an effective inhibitor of endothelial cell proliferation in culture (1, 2). These observations indicate that a solubilized extracellular domain of VEGI is responsible for its biological activity and hint at the necessity of a secretory mechanism for VEGI to effect its functions.

In this study, we describe two novel VEGI isoforms, VEGI-251 and VEGI-192, which show similar endothelial cell-specific expression as VEGI-174. These alternatively spliced isoforms differ in their N-terminus regions. VEGI-251, the most abundant isoform, possesses

unique secretory properties, and is detectable in cell conditioned media. It causes endothelial cell apoptosis if overexpressed in these cells. VEGI-251 also inhibits xenograft tumor growth if overexpressed in cancer cells. These findings suggest that VEGI-251 is an endothelial cell-secreted inhibitor of angiogenesis.

BODY

MATERIALS AND METHODS

Northern blotting

Multitissue Northern blots and multitissue dot-blot panels (Clontech, Palo Alto, CA) were hybridized in ExpressHyb solution (Clontech) with double-stranded cDNA probes. The full-length VEGI-174 probe was a Hind III-BamH I cDNA fragment (GenBank Accession #AF039390) in pcDNA3.1 (Invitrogen, Carlsbad, CA). For isoform-specific probes, a 297-bp VEGI-251 template encoding its N-terminal 99 amino acids was made by polymerase chain reaction (PCR) amplification and labeled with ^{32}P-dCTP by random priming (Invitrogen, CA). The VEGI-174 specific probe corresponding to its N-terminal 22 amino acids was made by end-labeling a 66-bp PCR product. The blots were hybridized at 42°C overnight and washed in wash buffer 1 (2 × SSC, 0.1% sodium lauryl sulfate) and wash buffer 2 (1 × SSC, 1% SDS) at 42°C followed by autoradiography at –70°C with an intensifying screen.

VEGI isoform cloning

We amplified human VEGI 5'-sequences by using the technique of rapid amplification of cDNA ends (RACE) from arrayed cDNA library panels (OriGene, Rockville, MD), according to the manufacturer's instructions. These panels contain cDNA samples prepared from 24 individual human tissues, with an adapter ligated to the 5' ends of the cDNAs. Two rounds of nested PCR were performed by using two pairs of oligonucleotide primers. In the first round of PCR, an adapter primer ADP1, 5' CGGAATTCGT CACTCAGCG 3', and a VEGI gene-specific primer

GSP1, 5'CCCGGATCCT ATAGTAAGAA GGCTCC 3', were used. The reaction products were then diluted 1:10 with water and used for the second round of PCR with another adapter primer, ADP2, 5'AGCGCGTGAA TCAGATCG3', and a VEGI gene-specific primer, GSP2, 5'CGGTGGATCC CGAGTTTGTC TCACAACTG3'. The PCR products were resolved on an agarose gel, purified, and sequenced on an ABI automatic sequencer.

Isolation of VEGI 251 and VEGI-192

Gene-specific primers, designed according to sequencing results of the RACE products, were used to repeat the second round PCR to confirm their sequence identities. Purified RACE products were then cloned into plasmid pCR3.1 (Invitrogen, Frederick, MD) and sequenced. Based on these sequences, isoform-specific PCR primers were designed to isolate corresponding full-length cDNA molecules from human cDNA libraries. The shared reverse primer Vg161 (161) 5'GTGTAATCCA CCAAAGAG3' was used with forward primers listed in **Table 1**.

Cell culture and cytokine treatment

Human umbilical cord vein endothelial cells (HUVE) and fetal bovine heart endothelial (FBHE) cells were obtained from Clonetics (Walkersville, MD) and grown in EGM-2 (Clonetics). Human dermal microvascular endothelial (HMVE), human coronary artery endothelial (HCAE) cells and NIH3T3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in EGM2-MV (Clonetics). Adult bovine aortic endothelial (ABAE) cells and mouse brain endothelioma bEND.3 were gifts from Peter Bohlen of ImClone Inc, New York, NY. Human coronary artery smooth muscle cells (HCASM) (Clonetics) and ABAE cells were cultured in IMEM (Biofluids, Biosource International, Camarillo, CA), 10% fetal bovine serum (FBS), and 1 ng/ml fibroblast growth factor-2 (Promega, Madison, WI). EA.Hy926, a human endothelial cell-derived hybridoma cell line, was a gift from Cora-Jean Edgell, University of North Carolina, Chapel Hill, NC. These cells, together with mouse brain bEND.3 and heart H5V endothelioma cell lines (ATCC), were maintained in IMEM with 10% FBS. Subconfluent cells grown in 100-mm dishes were treated with various doses of TNF-α (Biosource

International) or interferon γ (IFN-γ) (Roche Molecular Biochemicals, Indianapolis, IN) before RNA analysis.

Ribonuclease protection assays

For isoform-specific probes, cDNA fragments from human VEGI-174 (862–1062 bp), VEGI-251 (1–160 bp), VEGI-192 (277-656 bp) were generated by PCR and inserted between the EcoR I and Not I sites of pcDNA3 (Invitrogen) in the antisense direction. A mouse β-actin probe (824-942) was cloned into pSP72 (Promega) between the Hind III and BamH I sites. The VEGI and β-actin templates were linearized with Hind III and EcoR I, respectively. We synthesized antisense run-off probes with SP6 RNA polymerase by using the Maxiscript transcription kit (Ambion, Woodward TX). For nuclease protection with the RPAIII kit (Ambion), 15–20 μg of total RNA was hybridized overnight with 1–3 × 10^5 cpm of each probe at 52°C. We performed RNase digestion with 1:100 dilution of RNase A/T1 mix (Ambion) for 30 min at 37°C. The products of digestion were precipitated, resolved on a 6% polyacrylamide gel, and subjected to autoradiography at –70°C.

Gene structure analysis

We analyzed the organization of the human VEGI gene by PCR by using a bacterial artificial chromosome (BAC) clone (Genome Systems, Inc, St Louis, MO). PCR primers from exonic sequences were designed in order to generate overlapping PCR products. These PCR products were sequenced to determine their relative positions. Primers for the intronic region were designed based on the GenBank entry for Chromosome 9 Contig NT_017568, which correspond to sequences between bases 2,643,881 and 2,694,724. These are listed in **Table 2**. With human placenta DNA as a template, extra long PCR was performed by using an rTth XL PCR kit from Perkin Elmer (Foster City, CA) and the following extra-long PCR conditions: 95°C, 1 min, 97°C, 15 s, 60.5°C, 10 min, 17 cycles; 97°C, 15 s, 60.5°C, 10 min plus 15 s extension, 13 cycles, followed by final extension at 72°C for 11 min. The PCR fragments were generated with the primer pairs shown in **Table 2**. The PCR products were sequenced.

Production of monoclonal antibodies

Six-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously with purified recombinant VEGI protein (residues 29–174) at 50 μg per mouse in 0.1 ml of a complete Freud's adjuvant (Invitrogen). Following intraperitoneal boosters, mice with higher titers received a final intraperitoneal antigen injection of 30 μg/mouse. Spleen cells were isolated and fused with mouse myeloma SP2/O cells, by using polyethylene glycol 1500 (Roche Molecular Biochemicals, Indianapolis, IN). Hybridomas were selected by Hypoxanthine-Aminopterin-Thymidine medium and screened by ELISA. Positive hybridomas were cloned and the subclass of monoclonal antibodies was determined by using the mouse isotype kit (Sigma). Hybridomas were cultured on an INTEGRA CL 350 (INTEGRA Biosciences, Inc., Iiamsville, MD), the supernatants collected, and monoclonal antibodies were purified by AffiGel protein A agarose (Bio-Rad, Hercules, CA).

Production of polyclonal antibodies

Four- to six-month-old SPF New Zealand White rabbits (Charles River) were inoculated subcutaneously with 100 μg of recombinant VEGI mixed with complete Freud's adjuvant (Invitrogen). Following intramuscular boosters, serum was collected from rabbits showing substantial immune response. Because recombinant VEGI was produced in *E. coli*, the sera were purified by absorption with *E. coli* (transformed with an empty expression vector) cell lysate, then with human coronary artery smooth muscle cell lysate.

Analysis of VEGI in mammalian cells and conditioned media

The full-length VEGI-251 coding region was inserted between the Hind III and BamH I sites of pcDNA3 (Invitrogen). These pcDNA3 plasmids, including vector, were transfected into MDA-MB-231 breast cancer cells by electroporation. Stable transfectants were selected in 2 mg/ml G418 sulfate (Invitrogen). Conditioned media were concentrated 50-fold with Centricon filters (MW cutoff 10,000). Both cell lysates and concentrated conditioned media were immunoprecipitated with protein A/G agarose (Oncogene, Boston, MA) and the polyclonal

antibody against VEGI. The samples were analyzed by Western blotting. Detection was effected with a 1:1,000 dilution of mouse monoclonal antibodies 1-8F, and visualized with anti-mouse IgG antibody conjugated with horseradish peroxidase (ECL kit, Amersham).

Lentiviral gene transfer and apoptosis assay

The lentiviral vectors containing VEGI-174, sVEGI, and VEGI-251 were prepared by using previously described methods (15–18). Briefly, the lentiviral vector was generated in 293T cells with three plasmids: the transducing plasmid pHR'CMV-VEGI, the packaging plasmid pCMV ΔR8.2 Δvpr, and the envelope plasmid pCMV-VSV-G. Viral supernatants were collected every 24 h after two days post-transfection, purified by 0.45 μm filtration, and titrated by p24 assay (15–18). One pg of p24 count was defined as one tissue culture infective dose (TCID). For cell toxicity assays, HUVE cells were plated at a density of 2×10^4 per well in a 24-well plate 20 h before infection with viral supernatant. The cell number was expected to double 20 h after plating. Increasing doses of viral vector were added to HUVE cells. The multiplicity of infection (MOI) was estimated as TCID per cell at the time of infection. The number of adherent cells remaining in culture 24 h after viral infection were determined by Coulter Counter.

For apoptosis, TUNEL assay was performed 24 h after infection with viral supernatant, according to the procedure described by the manufacturer (fluorescein in situ cell detection kit, Roche Molecular Biochemicals). Efficiency of transduction was assessed by fluorescence microscopy using red fluorescent protein (Clontech).

In vivo tumorigenicity assay

Cells from stably transfected MDA-MB-231 clones containing empty pcDNA3 vector, VEGI-174, VEGI-251, or sVEGI were injected into mammary fat pads of female athymic nude mice (1×10^6 cells/injection). Tumor volumes were monitored in a blinded manner. Determination of microvessel density was carried out as described (19). Briefly, intratumor microvessels were immunostained with rat anti-mouse CD31 (PECAM-1) monoclonal antibody (clone MEC13.3, Pharmingen International, San Diego, CA). The antibody was diluted 1:100 in PBS, incubated

overnight with 5 μm paraffin-fixed tumor sections, and visualized with a biotinylated anti-rat IgG antibody (Vector Laboratories) by the Vectastain ABC method (Vector Laboratories). The sections were examined under low power (×10 objective lens and ×10 ocular lens) to identify the most vascular areas of the tumor ("hot spots", see reference (19)). Within these areas, a maximum of 10 fields at ×400 magnification (×40 objective and ×10 ocular lens; 0.16 mm^2 per field) were examined, and the mean values calculated. Large vessels with muscular walls were excluded. The lumen was not required to identify a vessel. Any positively stained endothelial cells or cell clusters, clearly separate from adjacent microvessel, tumor cells, and connective tissue elements, were regarded as distinct countable microvessels. All measurements were performed in a blinded manner. The results were analyzed by ANOVA. The *a priori* level of significance was set at $P<0.05$.

RESULTS

Detection of multiple VEGI transcripts and cloning of novel VEGI isoforms

Previous studies of VEGI-174, which is believed to be membrane-bound, had shown apoptotic activity of its C-terminal peptide on endothelial cells, but overexpression of the full-length VEGI-174 was ineffective on xenograft tumors formed by transfected cancer cells. Because the secretable recombinant sVEGI inhibited tumor growth, we postulated that the VEGI gene may express alternate forms in human tissue.

With a full-length cDNA of the originally discovered human VEGI as a probe, Northern blots of normal human tissues consistently reveal multiple bands of the following sizes: 7.5kb, 2.0 kb, and 1.5 kb (**Fig. 1**). These multiple transcripts for VEGI show somewhat overlapping tissue distribution and demonstrate the existence of a VEGI family. To elucidate the structure of the VEGI-related transcripts, we undertook the isolation of VEGI isoforms by PCR. With VEGI-specific 3' primers, 5'-RACE was used to amplify 5' ends of VEGI messages from a number of human tissues. The products were then cloned into pCR3.1 and sequenced (**Fig. 2**). Sequence analysis reveals two novel VEGI sequences, VEGI-251 and VEGI-192 (**Table 1** and **Fig. 2**). Based on these 5' sequences, isoform-specific primers were designed and full-length cDNA

clones were isolated from cDNA libraries. Three VEGI isoforms were thus isolated from fetal brain and adult uterus and lung (**Fig. 2A** and **B**). The novel cDNAs contain open reading frames of 251 and 192 amino acids (**Fig. 2B**), with calculated molecular weights of 28,086 and 21,952 Dalton respectively. The two novel VEGI peptides, VEGI-251 and VEGI-192, share the carboxyl 151 amino acid residues with the original VEGI (1), now referred to as VEGI-174. The hydropathic profile of the proteins indicated a hydrophobic region of 20 amino acid residues in VEGI-251 near its N-terminus (**Fig. 2B**), which is absent from VEGI-174 and VEGI-192. This sequence is predicted to comprise a signal peptide. Another highly hydrophobic, possibly transmembrane region, was previously identified at the N-terminus of VEGI-174 (**Fig. 2B**).

Expression of VEGI isoforms

The individual expression patterns of the isoforms was examined further by Northern analysis with multitissue blots. A 7.5 kb VEGI-251 transcript was detected in placenta, kidney, lung, and liver, whereas the 2 kb VEGI-174 transcript was observed in liver, kidney, skeletal muscle, and heart (**Fig. 3**). When these same probes were used on a multitissue RNA dot blot, it was observed that, in addition to overlapping expression between VEGI 251 and 174 in prostate, salivary gland, and placenta, VEGI-251 was more abundant than VEGI-174 in fetal kidney and fetal lung, whereas VEGI-174 is more abundant in heart, skeletal muscle, pancreas, adrenal gland, and liver (**Table 3**). The significance of such expression patterns is at present not readily apparent to us. VEGI-192 mRNA could not be detected by Northern blotting probably due to low abundance.

VEGI expression *in vitro* was also examined by RNase protection assay. In agreement with previous observations for VEGI-174, VEGI-251 and VEGI-192 were detected in the same cell types as VEGI-174, which are present in human endothelial cells, including HCAE, HUVE, and HMVE cells and undetectable in HCASM, ABAE, and mouse endothelioma bEND.3 (**Fig. 4**). It should also be noted that more than one isoform is expressed in the same cell type, with VEGI-251 being the most abundant. This finding suggests that the expression of these isoforms plays a regulatory role in VEGI function.

Human VEGI gene organization

To determine the structural relationship of the three VEGI transcripts, we analyzed the organization of the human VEGI gene. This was done with a BAC clone, as well as with genomic DNA isolated from human placenta samples. We found that the human VEGI gene spans over 17 kb, with 4 exons and 3 introns (**Fig. 5**). The intron-exon junctions conform to the GT-AG rule. Based on the size of fragment 2, intron 1 is estimated at 13-15 kb, although sequence information could not be obtained from this PCR product. All three isoforms share a common 438 bp region encoding residues 29-174 of VEGI-174 encoded by exon IVb (**Fig. 5**), but their 5' regions are generated from alternative exon usage. VEGI-251 and VEGI-192 use exonic splice acceptor sites to generate their respective products (**Table 4**). Ribonuclease protection and 5'RACE studies with genomic probes and HUVE RNA indicate that the putative transcription initiation site for VEGI-251 is located about 100 bp upstream of its ATG (unpublished observations, Chew, L. J.) but those for VEGI-174 and VEGI-192 have yet to be mapped. Due to the very low abundance of VEGI-192 RNA, subsequent studies have focused on VEGI-251. Although it is presently unclear whether all the isoforms initiate at the same promoter, we nonetheless reasoned that the significance of generating multiple transcripts could lie in differential regulation of synthesis, which in turn may point toward the relative importance of one particular VEGI isoform.

VEGI isoform transcripts are modulated in parallel by cytokines

To test for the possibility of differential regulation in VEGI isoform transcription, we analyzed VEGI gene regulation by using an anti-angiogenic paradigm of TNF-α treatment. Although many studies describe antiproliferative effects of proinflammatory cytokines such as TNF-α on endothelial cells, these cytokines can also be angiogenic depending on dose and system used (see Discussion). Such modulatory effects on endothelial cells may serve to regulate the levels of an endothelial cell-specific cytokine such as VEGI. We found that concentrations of 15 ng/ml of TNF-α or higher can induce an increase in VEGI RNA levels in both large vessel (umbilical vein) and small vessel (dermal microvascular) endothelial cells (**Fig. 6A** and **B**), and that all isoforms are induced in both these endothelial cell types. It is also clear that VEGI-251 remains the most abundant of the isoforms. The up-regulation of VEGI transcripts by TNF-α indicates

that VEGI-mediated activity is potentially a target of TNF-α action. In addition, the response of VEGI to another inflammatory cytokine, IFN-γ, showed a suppressive effect not only on basal VEGI levels, but also on TNF-stimulated VEGI expression (**Fig. 6C**). Both these paradigms, which demonstrate specific effects of distinct cytokines, affect all VEGI isoform transcripts in parallel. This finding indicates that the acute control of VEGI function through the synthesis of multiple peptides most likely lies at the post-transcriptional level.

Demonstration of VEGI in cell-conditioned media

Given the hydrophobic residues in the N-terminus of VEGI-251 and preliminary results showing plasma membrane localization of an N-terminally fluorescent-tagged VEGI-251, it appears likely that VEGI-251 is a secreted protein. To test this hypothesis, we generated stable transfectants of VEGI-251 in MDA-MB-231 breast cancer cells. As a negative control, transfectants of pcDNA3 vector were also made. Expression of the constructs in MDA-MB-231 was confirmed by RNase protection assay (not shown). It should be noted that the survival and proliferation of these cells in vitro was not affected by either vector or VEGI transfection (data not shown). The conditioned media of the MDA-MB-231 transfectants was collected, concentrated, and immunoprecipitated with a polyclonal VEGI antibody and subjected to Western analysis with an anti-VEGI monoclonal antibody 3-12D. Our results revealed a protein of molecular weight of about 25 kD (**Fig. 7A**). The appearance of the doublet cannot be readily explained, but may be the result of alternate glycosylation or other post-translational modification of the recombinant peptide in transfected MB231 cells. No VEGI protein was detected in media from untransfected cells (not shown) or cells bearing the empty pcDNA3 vector (**Fig. 7A**). VEGI-174 could not be detected in conditioned medium under similar experimental conditions (not shown). In a separate experiment, Western analysis of concentrated HUVE cell-conditioned medium also revealed a band of similar molecular weight as that obtained with VEGI-251 transfectants, as did HUVE cell-conditioned medium immunoprecipitated with the polyclonal antibody (**Fig. 7B** and data not shown). Note that endogenous VEGI protein is undetectable in HUVE cell lysate (**Fig. 7B**). These observations indicate that VEGI-251 is not membrane-bound but is instead a secreted protein.

Secretion of VEGI by endothelial cells causes apoptotic cell death

The low endogenous VEGI levels in HUVE cells made it necessary to find an alternate system for functional studies. To test the biological activity of VEGI-251 on endothelial cells, we chose to use a lentivirus gene delivery system to transfect HUVE cells with VEGI expression constructs. Our observations with VEGI-174, VEGI-251, and sVEGI, a secreted form of recombinant VEGI consisting of residues 23-174 of VEGI-174 and a secretory signal peptide derived from interleukin-6 (1), show that only the secreted forms of VEGI, including VEGI-251 and sVEGI, were cytotoxic to HUVE cells (**Fig. 7C**), whereas VEGI-174 was without effect. The lentiviral gene delivery was highly effective, as indicated by the use of a red fluorescent protein construct, which confirmed that more than 90% HUVE cells could be transduced (**Fig. 7D**). These results indicate that HUVE cells bear membrane receptors for VEGI that can become activated via an autocrine mechanism. The mechanism of cell death was investigated further by TUNEL assay. In contrast to vector-transfected cells, VEGI-251-overexpressing cells were found to be apoptotic, as evidenced by abundant fluorescent dUTP end-labeling (**Fig. 7D–G**).

Antitumor activity of VEGI-251

It has been shown previously that sVEGI, the secreted form of recombinant VEGI, was effective in inhibiting the growth of MC38 colon carcinoma tumors in vivo (1). Because native VEGI-251 is a secreted protein, we determined whether it could also inhibit the growth of human xenograft tumors in vivo. Stable MDA-MB-231 clones of VEGI-251, VEGI-174, and sVEGI transfectants were selected. Cells from each clone were injected into mammary fat pads of female athymic nude mice. Tumor growth rates were determined. Pooled vector-transfected clones were used as controls. Untransfected parental cells were also used as a control and found to be identical with the vector-transfected clones. Our results show that overexpression of full-length VEGI-174 by cancer cells had little effect on the growth of the xenograft tumors (**Fig. 8A**). However, overexpression of the intact VEGI-251, as well as the sVEGI fusion protein, retarded tumor growth significantly. These observations are in good agreement with the effect of lentiviral transfection in vitro (**Fig. 7C**), which confirms the biological activity of native VEGI-251.

We then determined the effect of full-length VEGI-251 overexpression by cancer cells on tumor neovascularization. Tumor microvessel density was found to be significantly reduced with the expression of VEGI-251 (**Fig. 8B**). The extent of reduction was comparable with that in sVEGI overexpressing tumors. Because the sVEGI fusion protein consists of residues 23–174 of VEGI-174, the results indicate that residues 23–174 contained the biological equivalent of native VEGI-251. Taken together, these findings demonstrate that secretion of VEGI-251 into the extracellular matrix is necessary for its antitumor activity. In addition, similar to sVEGI, this antitumor activity of VEGI-251 is not due to a direct effect on tumor cells but rather to interference with the development of tumor-associated vasculature.

DISCUSSION

It has been shown previously that recombinant VEGI comprising residues 29–174 causes growth arrest and apoptosis in ABAE and HUVE cells (1–3, 5), whereas full-length VEGI-174 is inactive on xenograft tumor growth when overexpressed in the cancer cells (1). These may be ascribed to insoluble recombinant protein or because it is retained within transfected cancer cells. A recombinant secreted form of VEGI-174, sVEGI, can inhibit tumor growth when overexpressed in cancer cells, which suggests that release into extracellular matrix is essential for anti-tumor activity of VEGI (1). Although VEGI-174 does not elicit endothelial cell arrest or apoptosis via an autocrine or paracrine mechanism, we cannot rule out the possibility that it may possess other, as yet uncharacterized, activities. Whereas these earlier studies are important for defining the active domain of VEGI-174 for anti-angiogenic cancer intervention, the question of physiological role for this particular VEGI gene product remains unanswered.

Our observations of multiple VEGI transcripts in human tissue led us to propose the existence of a secreted VEGI isoform. VEGI-251 was found to be 7.5 kb on our Northern blots, but a previous report of VEGI-174 described a transcript of the same size. In our hands, a VEGI-174-specific probe detected a 1.5 kb RNA instead. It is possible that in the earlier report (1), the 7.5 kb message detected with the full-length VEGI-174 probe was in fact VEGI-251 because of its greater abundance. Nonetheless, it is interesting that a large UTR is associated with a relatively small open reading frame of 753 bp. Indeed, a number of TNF-induced messages also possess

unusually long UTRs (20). Given the established roles of UTRs in message stability and translational efficiency (21–23), it would be reasonable to expect either function to explain the long UTR and high basal levels of VEGI-251 RNA in HUVE cells compared with other variants.

It is now well established that the process of alternative splicing is important in regulating gene function during development, in response to exogenous growth factors and cytokines (24–31). VEGI-251 RNA is noticeably high in fetal organs, demonstrating differential expression during development, and the prevalence of VEGI-251 in vitro in unstimulated and TNF-stimulated adult endothelial cells strongly suggests the importance of this alternatively spliced product. Further, the preferential expression of VEGI-251 over VEGI-174 in fetal kidney and lung seems to suggest involvement in early vascular development. Because the 151-residue active domain shared by all the isoforms possesses antiproliferative as well as apoptotic activity on endothelial cells, this finding of differential expression may indicate a complex role for VEGI-251 not only in the termination but also initiation and progress of angiogenesis.

There is an increasing number of peptides found to exist as both membrane-bound and soluble forms as a result of alternative splicing. A striking example is that of soluble receptor of VEGF, sFlt-1, which serves as an antagonist of the pro-angiogenic VEGF (32). Members of the TNF family, such as TNF-α (33) and Fas ligand (34–36), notably exist as transmembrane and soluble forms that elicit distinct, and sometimes opposing, effects on inflammatory and apoptotic activities of TNF. Based on its primary sequence, VEGI-174 was predicted to be a type II membrane-bound protein that displays a single transmembrane stretch separating a short N-terminal cytoplasmic region from a C-terminal extracellular domain. The C-terminus contains the active domain, evidenced by anti-angiogenic activity of a truncated 29–174 (1–3, 5).

Evidence for the secretory nature of VEGI-251 is provided by detection of VEGI peptide in conditioned media of stably transfected cells and of unstimulated vascular endothelial cells. Because endogenous VEGI protein in cell lysates is not consistently detectable by Western analysis with our antibodies, it is possible that only very low levels are synthesized or that the majority of the protein synthesized is not retained but is instead secreted. In this respect, we have observed that a large portion of HUVE cells often undergo spontaneous cell death at confluence.

Our analysis of VEGI mRNA has revealed a relatively low level in proliferating HUVE cells and a maximum at confluence (37). Because ABAE cells, which express a much lower level of VEGI, do not undergo apoptosis under normal culture conditions when confluent, it is possible that endogenous VEGI activity may be associated with density-dependent HUVE apoptosis in vitro.

Because VEGI gene expression is rapidly up-regulated by TNF-α and overexpression of VEGI-251 inhibits endothelial cell growth and reduces microvessel density in xenograft tumors, it is possible that VEGI may mediate at least some of the effects of TNF-α in endothelial cells. TNF, a potent multifunctional hormone produced predominantly by macrophages, can both inhibit and stimulate angiogenesis in a dose-dependent manner, with high doses inhibiting and low doses stimulating (38, 39). It was also reported to induce regression of tumors in animals (40). TNF blocks VEGF- and bFGF-stimulated endothelial cell proliferation (41, 42). Indeed, there is now evidence that an inverse relationship exists between TNF-α and tumor-associated microvessel count in non-small-cell lung carcinoma (43), which suggests a favorable effect of TNF-α levels on clinical outcome. We have also recently described a dual mechanism of antiproliferative activity for VEGI in primary endothelial cells: VEGI prevents G_0/G_1 cells from re-entering the cell cycle in response to growth stimuli, whereas it induces caspase 3-mediated apoptosis in actively proliferating cells (37). It is thus possible that synergy between TNF and VEGI may be involved in regulating endothelial cell function.

INF-γ is known to enhance TNF-mediated endothelial cell activation (44) and to synergize with TNF in the induction of apoptosis (45–47). In light of these reports, we therefore find it somewhat surprising that IFNγ should antagonize both basal and TNF-stimulated VEGI gene expression in vitro. Perhaps during endothelial cell damage and inflammation, which result in elevated levels of TNF and IFN, other cytokines are produced, which serve to influence endothelial cell viability by altering the balance of regulatory molecules that induce or suppress apoptosis (48). Further studies will be required to address the seemingly complex relationship between cytokine induction and VEGI action.

The VEGI isoforms described here provide evidence that VEGI exists as an intracellular form, as well as a secreted form. Our observations indicate that the secretory nature of VEGI-251 accounts for its anti-angiogenic activity in vivo. These findings also lend support to the physiological relevance of this unique gene. Although studies on VEGI have thus far focused on endothelial cell activation and apoptosis, we do not exclude the possibility that VEGI may also exert immunomodulatory effects on other cell types. Future clarification of distinct functions exerted by the various forms of VEGI will be important for understanding the angiogenic process and will be instrumental in assessing the potential utility of VEGI peptides as a therapeutic agent.

KEY RESEARCH ACCOMPLISHMENTS

1 – Identified two new isoforms of VEGI
2 – Determined the structure of the VEGI gene
3 – Demonstrated that VEGI-251 is a secreted protein
4 – Demonstrated that VEGI-251 is an inhibitor of angiogenesis and tumor growth

REPORTABLE OUTCOMES

All results described here are reportable.

CONCLUSIONS

Vascular endothelial cell growth inhibitor (VEGI), a member of the TNF family, is an endothelial cell-specific inhibitor of angiogenesis. Overexpression by cancer cells of a secretable VEGI fusion protein resulted in abrogation of xenograft tumor progression, but overexpression of full-length VEGI was completely without effect. This finding indicates that secretion is essential for VEGI action. Here we report the identification of two new VEGI isoforms consisting of 251 and 192 amino acid residues. Both isoforms show endothelial cell-specific expression and share a C-terminal 151-residues segment with the previously described VEGI, which comprises 174 residues. The isoforms are generated from a 17 kb human gene by alternative splicing. Their expression is regulated by TNF-α and interferon-γ. VEGI-251, the

most abundant isoform, contains a putative secretion signal. VEGI protein is detected in conditioned media of endothelial cells and VEGI-251-transfected mammalian cells. Overexpression of VEGI-251 in endothelial cells causes dose-dependent cell death. VEGI-251-transfected cancer cells form xenograft tumors of reduced growth rate and microvessel density compared with tumors of empty vector transfectants. These findings support the view that VEGI may function as an autocrine inhibitor of angiogenesis and a naturally existing modulator of vascular homeostasis.

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APPENDICES

Tables and Figures attached.

Table 1

Unique 5'-sequences of human VEGI isoforms. Forward PCR primers used to screen cDNA libraries to isolate full-length clones are underlined. The sequence of the shared reverse PCR primer is given in Materials and Methods.

Isoform	Source	Nucleotide sequence
VEGI-174	Brain	CAAAGTAATT TGCCCCAGGT CACTAGTCCA AGATGTTATT CTCTTTGAAC AAATGTGTAT GTCCAGTCAC ATATTCTTCA TTCATTCCTC CCCAAAGCAG TTTTTAGCTG TTAGGTATAT TCGATCACTT TAGTCTATTT TGAAAATGAT ATGAGACACT TTTTAAGCAA AGTCTACAGT TTCCCAATGA GAAAATTAAT CCTCCTCCTC TCTCGGGAAC
VEGI-251	Uterus	GGGGGGGGGG GTCAGAGGTG CCTGGTGTTG CTCCCCTTCC TTGCAGGACT CACCACATAC CTGCTTGTCA GCCAGCTCCG GGCCCAGGGA GAGGCCTGTG TGCAGTTCCA GGCTCTAAAA GGACAGGAGT TTGCACCTTC ACATCAGCAA GTTTATGCAC CTCTTAGAGC AGACGGAGAT AAGCCAAGGG CACACCTG
VEGI-192	Lung	CTCCTATCAT AGGCGCCATG CAACTCACAA AGGGCCGTCT TCATTTCAGT CACCCTTTGT CTCATACAAA GCACATTTCT CCTTTTGTTA CAGATGCACC TCTTAGAGCA GACGGAGATA AGCCAAGGGC ACACCTG

Table 2

Nucleotide sequence of oligonucleotide primers used in PCR analysis of the human VEGI gene. PCR product 2b was also sequenced using primers 61, 57, 51, and 53.

PCR product	Forward primer	Reverse primer
1	ATGGCCGAGG ATCTGGGACT	CTGCACACAG GCCTCTCCCTG
2a	50:CCTTGCAGGA CTCACCACAT ACCTGCTT	60:AGAGGCTAGG TTTCCAGTTA AACCCATTGA
2b	61:TGGGGATAAC CTTCAGCCTC ATATTTTTTA	52: ATGTGAAGGT GCAAACTCCT GTCCTTTTAG
2c	60:AGAGGCTAGG TTTCCAGTTA AACCCATTGA	61:TGGGGATAAC CTTCAGCCTC ATATTTTTTA
2d	57:GATCTGGAGG GACTGATGGA GAAGAAATGG	52:ATGTGAAGGT GCAAACTCCT GTCCTTTTAG
2e	51:TACGTGCCCA GTAGTGAGAT TGCTAGAC	52:ATGTGAAGGT GCAAACTCCT GTCCTTTTAG
2f	53:GAGGCTGATG AAAAGGAGAA CATAGCCATT ATT	52:ATGTGAAGGTGCAAACTCCTGTCCTTTTAG
3	CTAAAAGGAC AGGAGTTTGCA	CTGTAACAAA AGGAGAAAT
4	ATTTCTCCTT TTGTTACAG	CTTGAACAGG CACAGATGAAC
5	ATCTAGTTCA TCTGTGCCTGTTCA	GGGAGTTTGT CTCACAACTGG
6	CCAGTTGTGA GACAAACTCCC	GTAAGGCACA TGAAGTGTGAAAT

Table 3

Comparison of VEGI-174 and VEGI-251 RNA expression patterns determined by using human poly A-enriched RNA multitissue dot blot. Ranks: (-) undetectable; (±) not significantly above background; (+) very low; (++++) abundant. Ranking of the RNA signal intensity was carried out in blinded manner by three individuals. VEGI-192 expression was undetectable.

Tissue	VEGI-174	VEGI-251
Brain	++	-
Heart	++++	-
Aorta	+	-
Sk. Muscle	+++	-
Colon	++	±
Bladder	+	±
Uterus	+	-
Prostate	++	+++
Stomach	+++	++
Testis	+	-
Ovary	+	-
Pancreas	++++	±
Pituitary gl.	++	-
Adrenal gl.	+++	±
Thyroid gl.	+	-
Salivary gl.	++++	++++
Mammary gl.	+	±
Kidney	++	+++
Liver	++++	-
Sm. Intestine	+++	++
Spleen	++	±
Thymus	++	±
Peri. Leukocyte	+	-
Lymph node	+	±
Bone marrow	+	-
Appendix	+	+
Lung	++	++
Trachea	++	+
Placenta	++++	+++
Fetal brain	+	-
Fetal heart	+	-
Fetal kidney	+	++++
Fetal liver	++	-
Fetal spleen	+	+
Fetal thymus	+	-
Fetal lung	+	++++

Table 4

Organization of the human VEGI gene. Sizes of exons and approximate sizes of introns are indicated. Uppercase letters indicate exon sequence, and lowercase letters indicate intron sequence. Consensus splice junctions are underlined. Asterisk indicates the extreme 5' ends of VEGI-192 and VEGI-174 mRNA, which have not yet been identified; intron-exon junctions in these regions are therefore unknown.

EXON	size	5'splice donor	INTRON size	3' splice acceptor	EXON
I	310 bp	AGTTCAG gtaagccacatggca	~ 13 kb	Tttctttcccaatag GCTCTAAAAGGACA	II
II	43 bp	CACATCAGCAAGTTT gtaagtatgctcatc	~ 400 bp	*aatgtctagcacatc aaaagTATGCACTTCA TTTT	IIIa
IIIa	661 bp			CTCCTTTTGTTACAG ATGCACCT	IIIb
IIIb	48 bp	GGGCACACCTGACA gtaagcctccctgct	~458 bp	*ttcagattctatcag CCCTCTCTCTTTCTCT CCT	IVa
IVa	1092 bp			GGGCACACCTGACAGT TGTGAGA	IVb
IVb	1592 bp				

Fig. 1

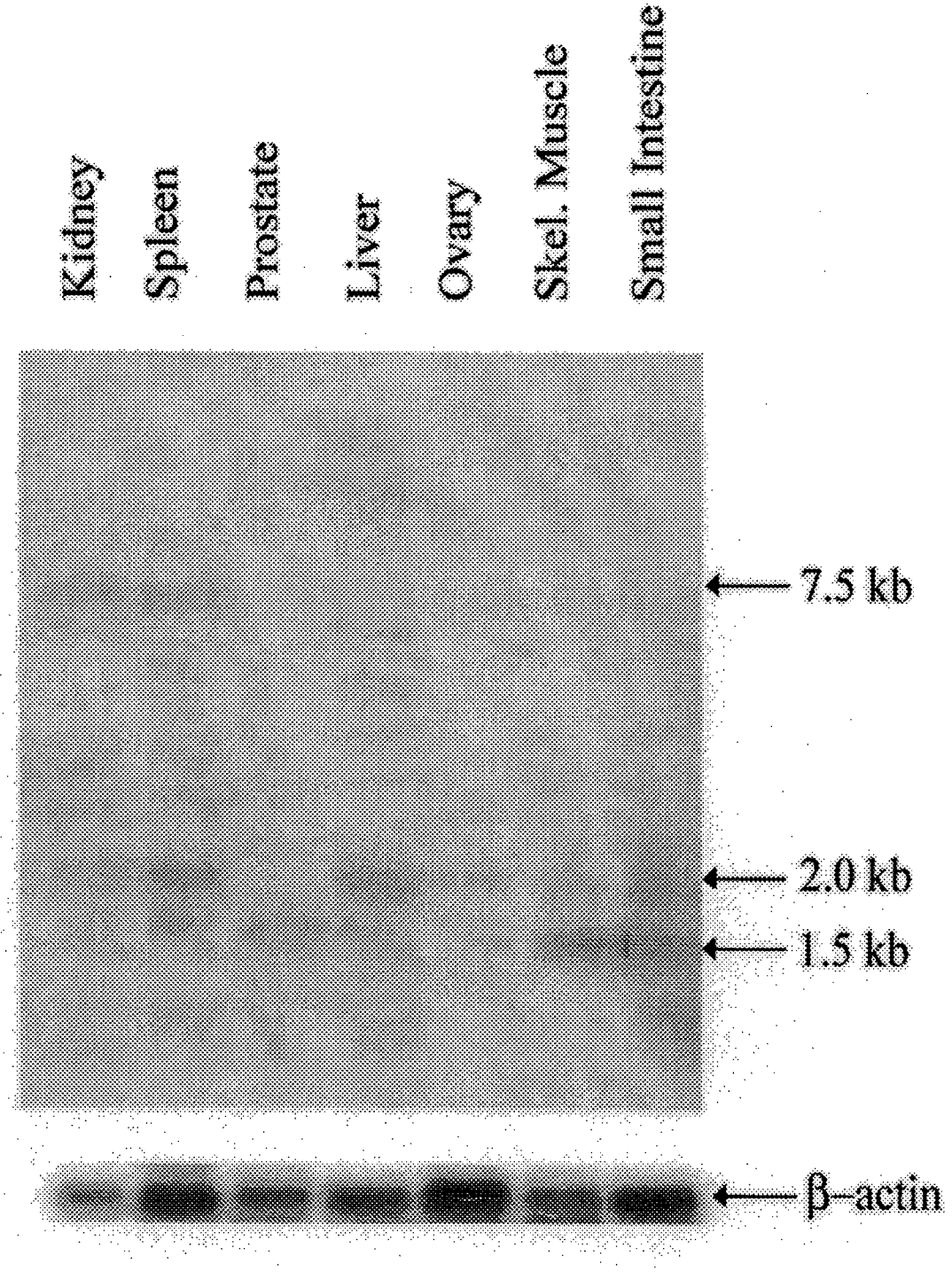
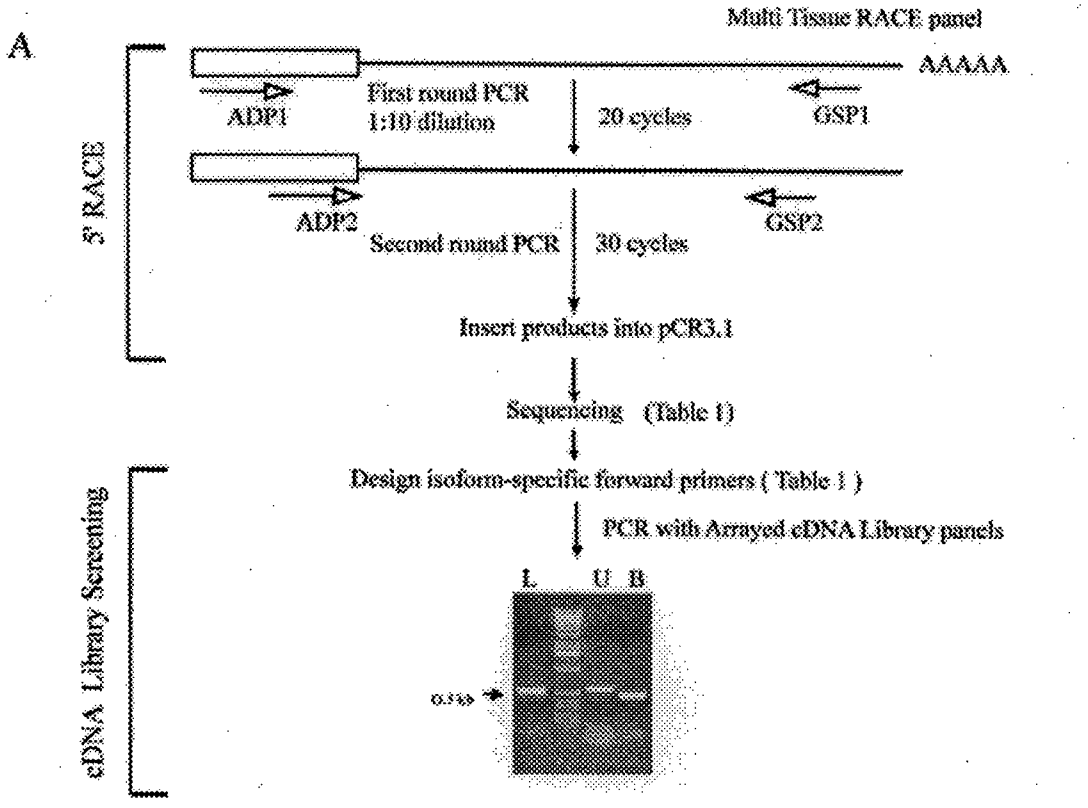


Figure 1. VEGI is expressed as multiple transcripts in human tissue. VEGI expression in adult human tissues was determined by multi-tissue Northern blotting analysis by using ^{32}P-labeled full-length VEGI-174 cDNA as a probe. Three distinct VEGI-related messages of the indicated sizes are detected as indicated.

Fig. 2



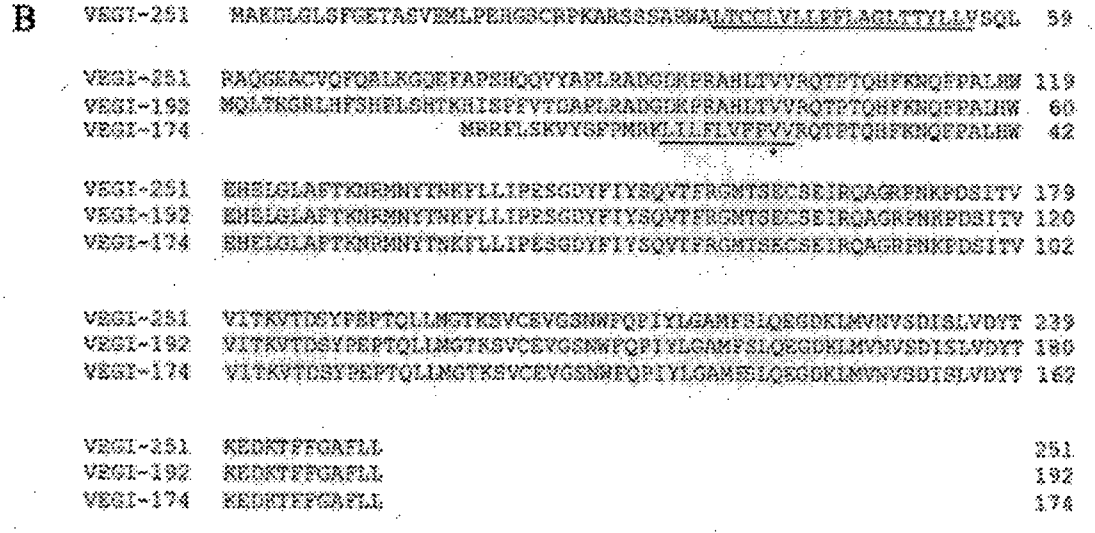


Figure 2. Isolation of novel VEGI cDNAs. A) Scheme showing synthesis of 5′ RACE products followed by cDNA library screening to isolate full-length VEGI cDNAs from various human tissues. Shaded boxes represent ligated 5' adapters present in the RACE panel. PCR primers are denoted by arrows with open arrowheads. PCR products of different sizes are visualized by ethidium bromide staining. The PCR products were isolated and subjected to sequencing. L, lung; U, uterus; B, brain. A 1-Kb DNA molecular weight ladder is shown between the L and U lanes. **B)** Amino acid sequence alignment of three VEGI isoforms. The putative hydrophobic regions of VEGI-251 and VEGI-174 are underlined. Asterisk denotes the start of homologous sequences.

Fig. 3

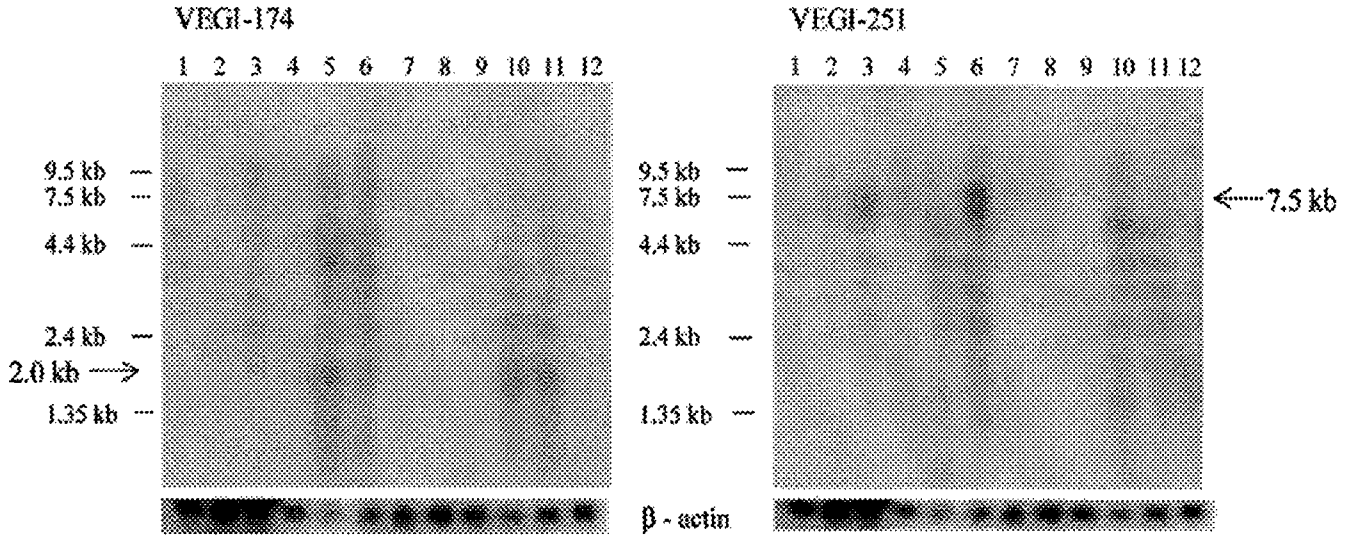


Figure 3. Differential expression of VEGI-174 and VEGI-251 in human tissues. Northern blotting analysis of VEGI expression in adult human tissues were performed with cDNA fragments specific for VEGI-251 and VEGI-174. A 2-kb transcript is detected with the VEGI-174 probe, whereas a 7.5-kb message is detected with the VEGI-251 probe (arrows). Equivalent loading was verified with human β-actin. The human tissues examined were as follows: 1) peripheral blood leukocytes; 2) lung; 3) placenta; 4) small intestine; 5) liver; 6) kidney; 7) spleen; 8) thymus; 9) colon; 10) skeletal muscle; 11) heart; 12) brain.

Fig. 4

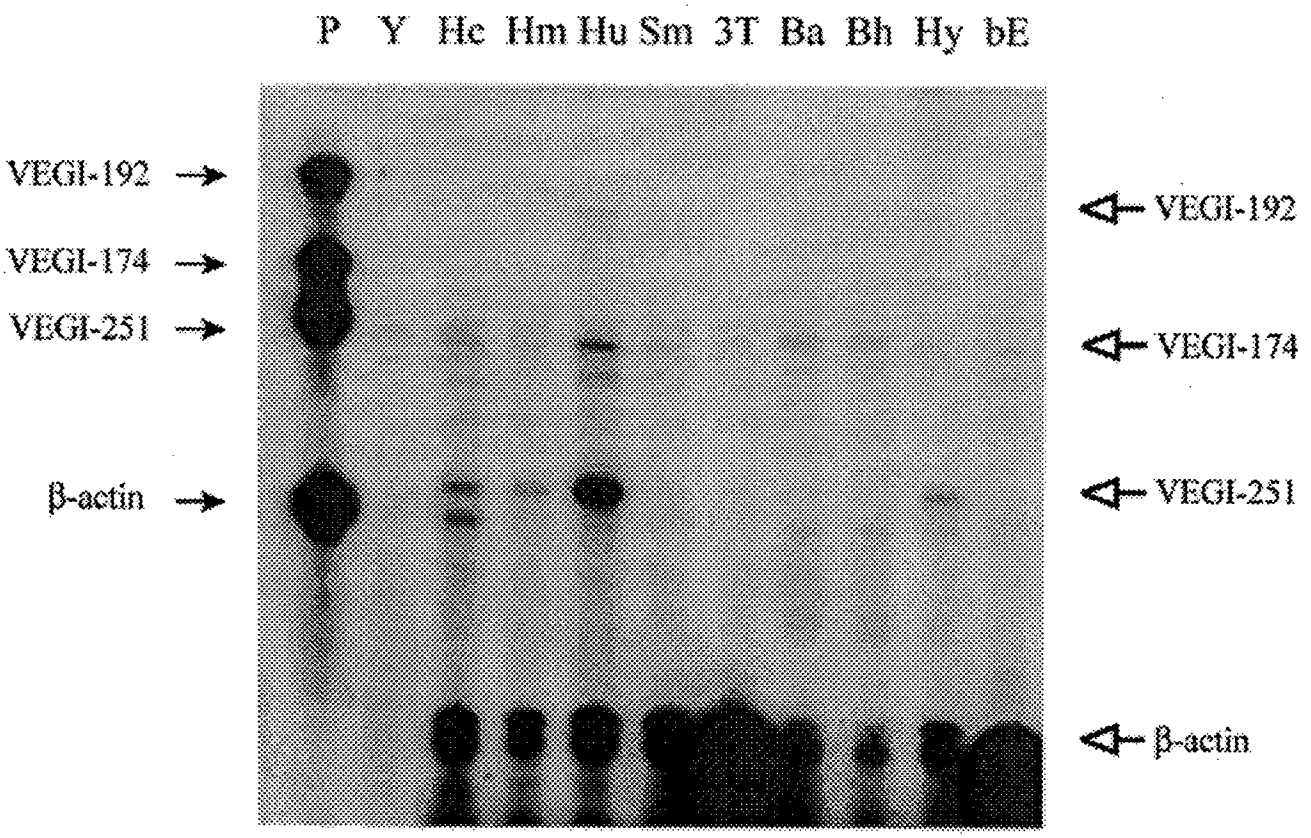


Figure 4. Ribonuclease protection analysis of VEGI isoforms in various cultured cells. Total RNA from each culture was hybridized with isoform-specific VEGI probes and β-actin for loading control. Full-length undigested probes are shown in the probes lane (P), indicated by solid arrowheads, and products of RNase protection are indicated by open arrowheads. Y, yeast RNA; Hc, human coronary artery endothelial cells; Hm, human dermal microvascular endothelial cells; Hu, human umbilical vein endothelial cells; Sm, human coronary artery smooth muscle cells; 3T, NIH3T3 mouse embryonic cell line; Ba, adult bovine aortic endothelial cells; Bh, fetal bovine heart endothelial cells; Hy, EA.Hy926 human endothelial hybridoma cell line; bE, bEND.3 mouse brain endothelioma cells.

Fig. 5

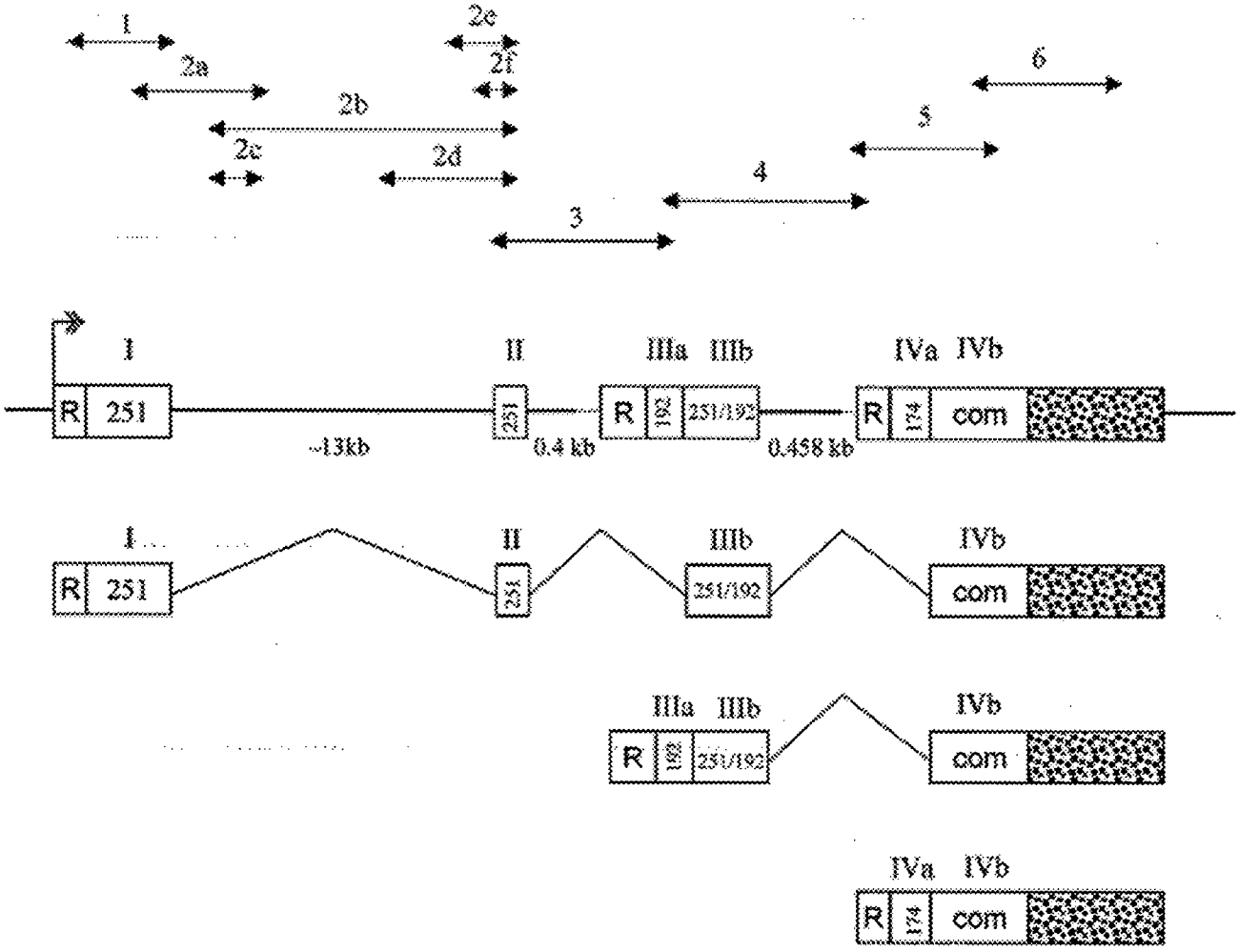


Figure 5. Gene structure of human VEGI and proposed generation of isoforms. The segments numbered 1 through 6 represent the PCR fragments generated during gene mapping, with specific primer pairs listed in Table 2. Boxes with roman numerals above represent exons and horizontal lines represent intronic sequence. The putative transcription start site is indicated by a double arrowhead. Letter R denotes the 5′ untranslated sequence unique to each respective transcript, and stippled boxes represent the common 3′ untranslated region. Approximate sizes of the introns are indicated. VEGI-251, VEGI-192, or VEGI-174 specific sequences are labeled '251', '192', or '174'. Exon IIIb encodes residues shared by both VEGI-251 and VEGI-192. The introns 5' of exons III and IV are dashed because the 5' ends or initiation sites of VEGI-192 and VEGI-174 transcripts have not yet been determined. 'COM' denotes the coding region of the last exon that is common to all three isoforms.

Fig. 6

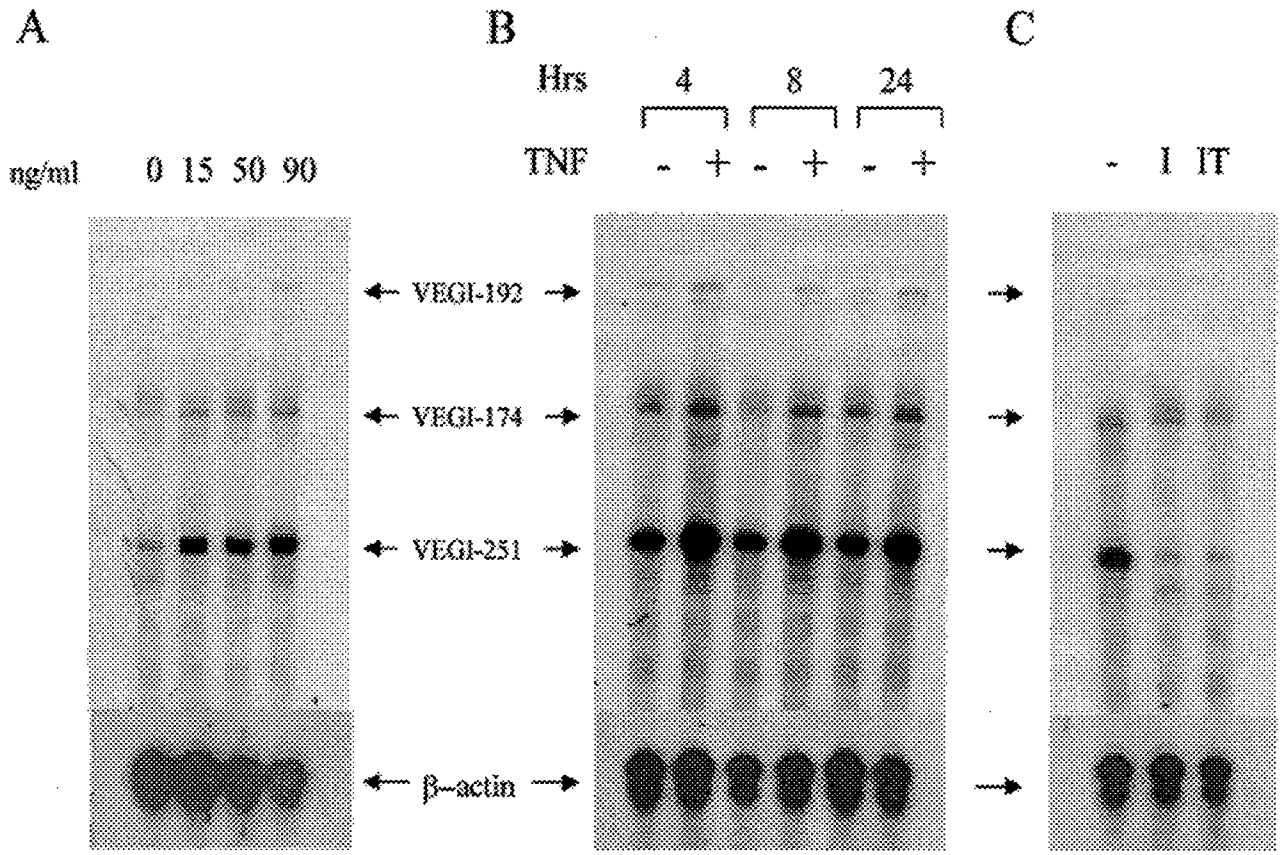


Figure 6. Regulation of VEGI isoform expression by TNF-α and IFNγ. Ribonuclease protection assays showing parallel induction of VEGI expression. Arrows indicate protected RNAs. **A)** HMVE cells treated with TNF-α at 15, 50, and 90 ng/ml over 24 h. **B)** Induction of VEGI gene expression by TNF-α in HUVE cells. HUVE cells were treated (+) with 20 ng/ml TNF-α for 4, 8, and 24 h. **C)** Down-regulation of basal and TNF-induced VEGI expression. HUVE cells were treated with 20 U/ml IFNγ in the absence (I) and presence (IT) of 80 ng/ml TNF-α for 24 h. Controls (-) received corresponding vehicle treatments.

Fig. 7

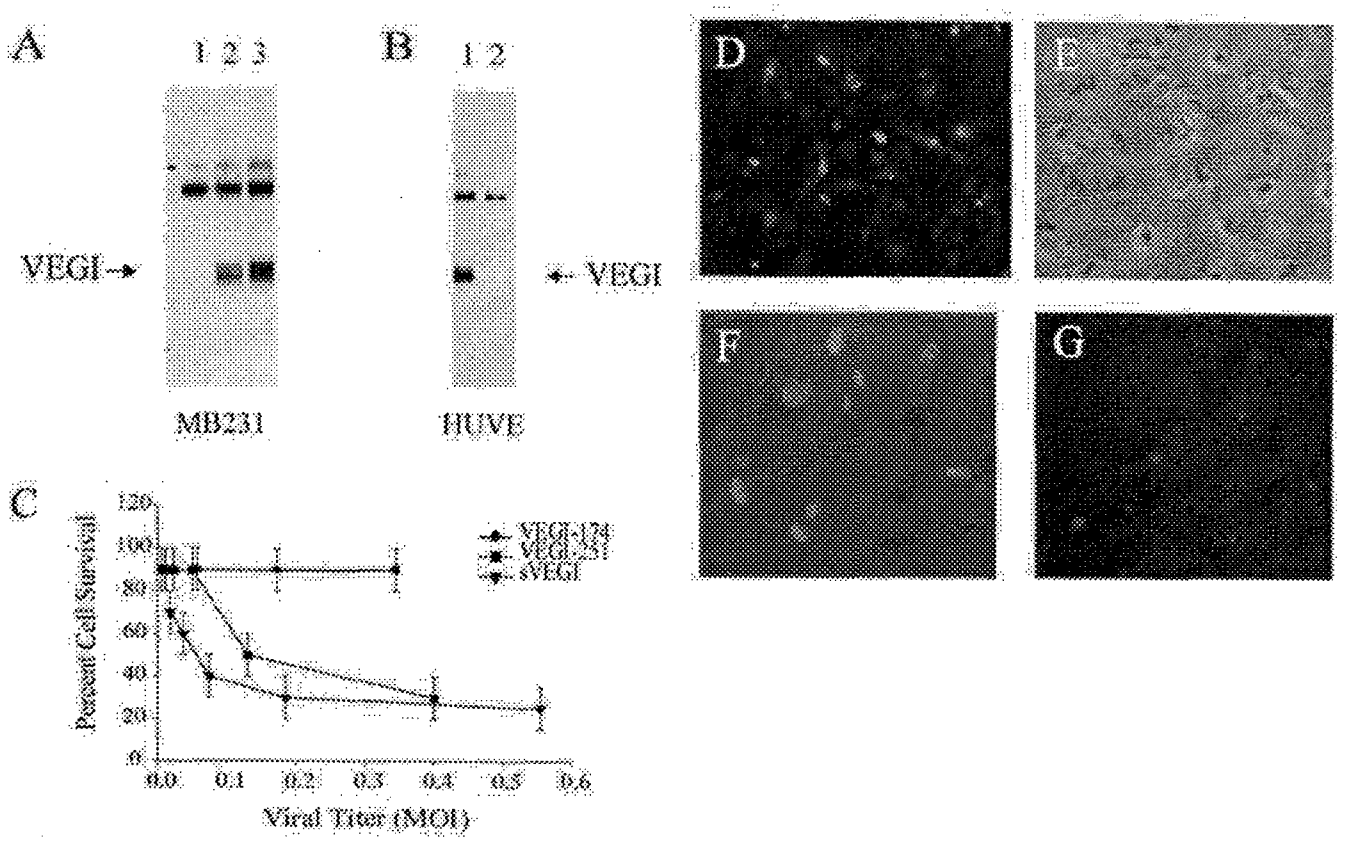


Figure 7. Secretion of VEGI-251 and endothelial cell apoptosis caused by VEGI-251 overexpression. A, B) Detection by immunoprecipitation and Western analysis of VEGI in VEGI-251-transfected MB231 cell or untransfected HUVE cell conditioned media. **A)** Conditioned medium from stable transfectants of MDA-MB231. Lane 1, pcDNA3 vector only, Lanes 2 and 3, two independent clones expressing VEGI-251. **B)** Lane 1, HUVE cell-conditioned medium, Lane 2, HUVE cell lysate. In both experiments, conditioned media were concentrated by 50-fold with Centricon filters (MW cutoff 10,000), immunoprecipitated by using polyclonal antibody, then subjected to SDS-PAGE, and Western detection by using monoclonal antibody 1-8F against the common C-terminal region of VEGI (residues 29-174). Both panels show VEGI peptides of approximately 25 kD. **C)** Lentivirus delivery of secreted VEGI is lethal to HUVE cells. Dose-dependent cytotoxicity of lentivirus carrying VEGI-251, sVEGI, or VEGI-174 is compared. Twenty-four hours following viral infection, adherent cells remaining in culture were counted by Coulter counting. Viral p24 levels were estimated, and viral dose is expressed as multiplicity of infection (MOI). Values shown are mean ± SE of three independent experiments. **D–G)** VEGI-251 overexpression causes apoptosis. **D)** Fluorescent image of HUVE cells infected with lentiviral Red Fluorescent Protein at 0.4 MOI (red, magnification ×100), showing that a large majority of the cells are infected. **E)** Phase contrast image of HUVE cells shortly after infection with lentiviral vector expressing VEGI-251. Most cells died 24 h postinfection. At 0.4 MOI, apoptosis (green, TUNEL assay) is readily observed in endothelial cells infected with VEGI-251 **(F)** but not with vector alone **(G)** **(E–G**: magnification ×400).

Fig. 8

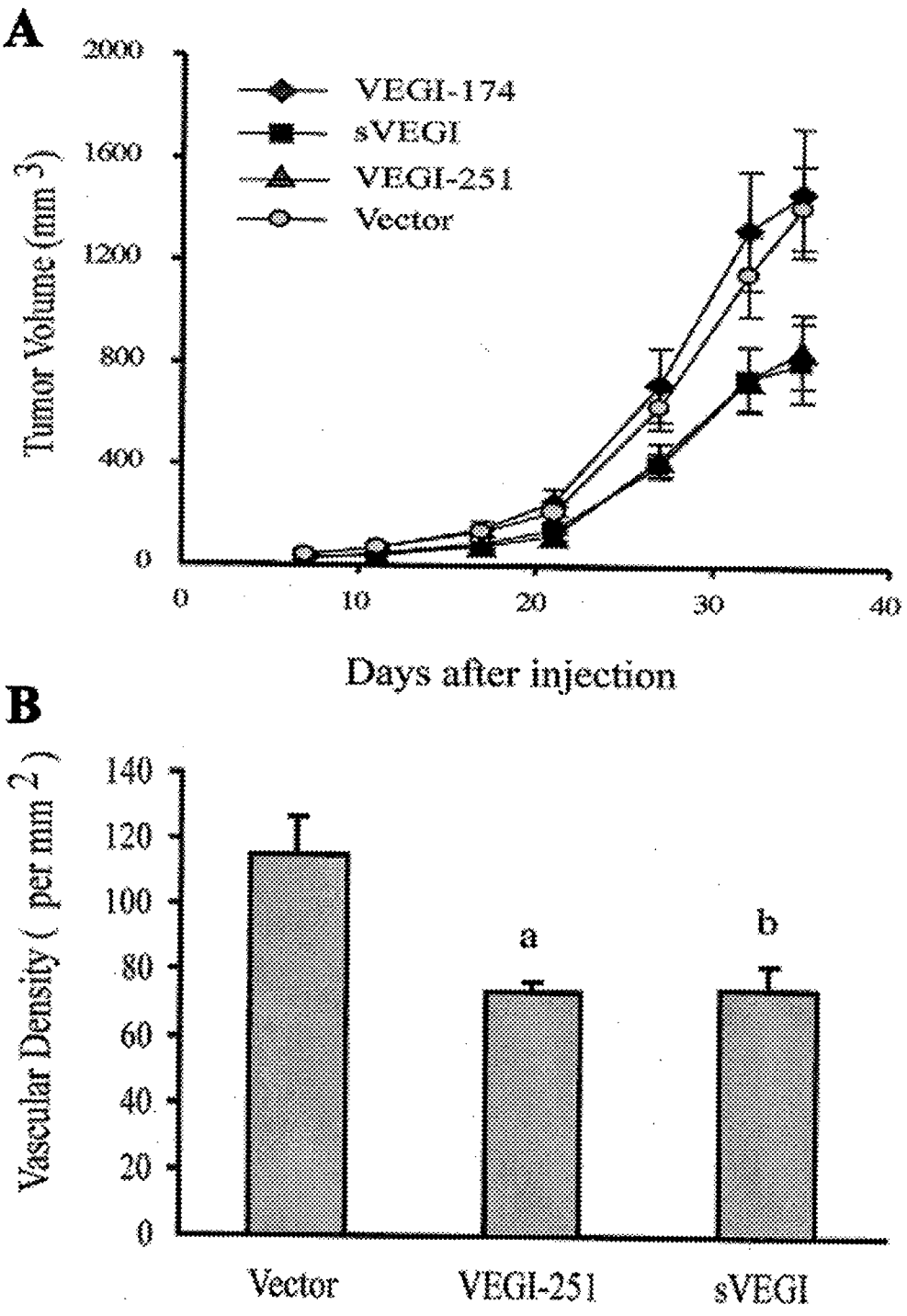


Figure 8. Overexpression of VEGI-251 retards tumor progression and interferes with tumor neovascularization. **A)** Retardation of xenograft MDA-MB-231 breast tumor growth by VEGI-251 or sVEGI. Three clones of stable transfectants of each of the indicated constructs were injected subcutaneously into mammary fat pads of female athymic mice, five animals per clone. Tumor volumes were monitored in a blind manner. Control mice received pooled pcDNA3 vector-transfected cells. **B)** VEGI-251 or sVEGI overexpression results in reduced microvessel densities in MDA-MB-231 xenograft tumors. Paraffin sections (5 μm) were from tumors described in (**A**). Vessels were identified by CD31 immunostaining. One-way ANOVA was used. a) $P < 0.0005$; (b) $P < 0.05$ vs. control xenografts with vector pcDNA3.